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Prodomain Processing of Recombinant Plasmepsin II and IV, the Aspartic Proteases of *Plasmodium falciparum*, Is *Auto*- and *Trans*-Catalytic

Yong Man Kim[1,*†], Mi Hyang Lee[2,*], Tae Gwang Piao[1], John Wha Lee[2], Jong Ho Kim[1], SungGa Lee[1], Kyung Min Choi[1], Jing Hua Jiang[1], Tae Ue Kim[3] and Hyun Park[1,‡]

[1]Department of Infection Biology, College of Medicine, Wonkwang University, 344–2 Shinyong-dong, Iksan, Jeonbuk 570-749, Korea; [2]College of Veterinary Medicine, Chonbuk National University, Jeonju, Korea; [3]Department of Biomedical Laboratory Science, College of Health Science, Yonsei University, Wonju, Korea

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Prodomain processing of the four food vacuole plasmepsins (PMs), the malarial aspartic proteases, is prerequisite for their activity on hemoglobin degradation of the parasite *Plasmodium falciparum*. Although previous studies have suggested the involvement of a calpain-like PM convertase in the processing of PMs, the underlying mechanism of their processing remains to be clarified. Here, to investigate the mechanism by which food vacuole PM II and IV are processed, we used their wild-type and mutant proteins in which the catalytic Asp residue in two active-site motifs was mutated, as well as protease inhibitors. Autocatalytic processing of wild-type PM II and IV was inhibited only by an aspartic protease inhibitor pepstatin A. Unexpectedly, their proteolytic activities were inhibited not only by pepstatin A but also by calpain inhibitor ALLN. The active-site mutants of both PM II and IV showed neither autocatalytic processing nor proteolytic activities. However, the mutants of both PMs were efficiently processed upon incubation with their respective wild type proteins. Furthermore, the mutants of both PMs were processed upon incubation with each other's wild-type PM in both pepstatin A- and ALLN-sensitive manners. These results suggest that the processing of PM II and IV occurs *via* an intra- and inter-molecular autocatalytic event as well as *via* a transcatalytic event between them.

Key words: active-site motif, aspartic protease, calpain, plasmepsin, *Plasmodium falciparum*.

Abbreviations: PM, plasmepsin; FV, food vacuole; ALLN, *N*-acetyl-L-leucyl-L-leucyl-norleucinal; SDS, sodium dodecyl sulfate.

It is estimated that nearly half of the world's population lives in malaria-endemic areas. Of the four species of *Plasmodium* responsible for human malaria, *Plasmodium falciparum* is the most lethal. A major reason for the persistence of malaria is the emergence of resistance to common anti-malarial drugs (*1*). Thus, it is important to identify new targets for antimalarial therapy and to develop drugs aimed at these targets (*2*).

Potential new targets for antimalarial chemotherapy include parasite enzymes required for the degradation of hemoglobin. The malaria parasite degrades hemoglobin as a source of nutrients in the erythrocytic stage, where it invades the host red blood cells (*3*). The hemoglobin degradation occurs in an acidic food vacuole (FV) formed by the parasite, and can result in up to 70% of host hemoglobin being consumed (*3*). In *P. falciparum*, three different classes of proteases, including aspartic proteases (Plasmepsins I–X) (*4*), cysteine proteases (Falcipains 1–3) (*5–7*), and metallo-proteases (Falcilysin) (*8*), are responsible for the hemoglobin degradation in a semiordered fashion (*3*, *9*). Plasmepsins (PMs) have been shown to play an important role in the initial cleavage as well as subsequent hydrolysis of hemoglobin (*4*, *10–12*). Since inhibitors of the PMs stop hemoglobin degradation in the FV and kill *P. falciparum* during *in vitro* culture, the enzymes have emerged as attractive targets for an antimalarial drug development (*9*, *13*, *14*).

In *P. falciparum*, ten genes are known to encode PMs or related enzymes (*14*). Of these, PM I, II, IV, and histo-aspartic protease (HAP) are known as food vacuole proteases have the capacity to digest hemoglobin (*4*). PM I, II, and IV are classical aspartic proteases with two aspartic acids in the active-site motif, whereas in HAP, one of the catalytic aspartic acids is replaced by histidine (*4*). Owing to their degradative role, it is essential that PMs only function where and when they are needed. Thus, they are synthesized as inactive precursors (zymogens), which are type II integral membrane proteins containing a transmembrane domain within their prodomains, and are converted into active enzymes by removal of the prodomains upon arrival at the FV (*4*, *12*, *15*, *16*). The importance of correct targeting of PMs to the FV for their processing is evidenced by the observations that the

*Yong Man Kim and Mi Hyang Lee contributed equally to this work.

†Present address: FCB-Pharmicell, #920 Sicox Tower, 513-14 Sangdeawong-dong, Sungnam, Kyungki-do 462-120, South Korea.
‡To whom correspondence should be addressed. Tel: +82-63-850-6769, Fax: +82-63-857-0342, E-mail: hyunpk@wonkwang.ac.kr

processing of PMs is blocked by treatment of parasites with brefeldin A, an inhibitor of anterograde protein traffic from the ER (*12*, *15*, *17*). As for the trafficking route of PMs to FV, it has recently been suggested that PM II is targeted to the FV through the secretory system of the cytostomal vacuole in a brefeldin A–sensitive manner (*17*), rather than a direct trafficking route from the ER to the FV (*18*). There is controversy over whether PMs can activate themselves to the mature form or if the activation occurs by virtue of other enzymes. Experiments with recombinant PM II and IV revealed that they were processed efficiently under acidic conditions in a pepstatin A–sensitive manner, like typical aspartic proteases (*15*, *16*, *19*). In contrast to autocatalytic processing, the requirement of a calpain-like protease in the processing of PMs has recently been implicated from the results that processing of PMs *in vivo* and *in vitro* was blocked by the calpain inhibitor *N*-acetyl-L-leucyl-L-leucyl–L-norleucinal (ALLN) but not by pepstatin A (*12*, *15*, *17*). Thus, understanding the molecular details of the processing of PMs may have implications for rational antimalarial drug design.

In this study, we have analyzed the processing of PM II and IV using their wild-type and catalytic Asp mutant proteins in the presence of protease inhibitors. Here, we report that the processing of PM II and IV occurs through not only an autocatalytic event but also a transcatalytic event between them, rather than through a separate processing enzyme.

MATERIALS AND METHODS

Materials—Pepstatin A, leupeptin, *trans*-epoxysuccinyl-L-leucylamido-4-guanidino-butane (E-64), 3,4-dichloroisothiocoumarine (DCI), and enzymes for DNA modification were from Roche (Mannheim, Germany). Phenylmethanesulfonyl fluoride (PMSF), *N*-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO).

Plasmids—The genes encoding the proform of PM II and IV (*4*, *20*) were generously provided from Daniel E. Goldberg (Department of Medicine and Molecular Microbiology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO). Site-directed mutants in which the aspartic acid residue at two active-site motifs of PM II (D158A and D338A) and IV (D155A and D335A) were replaced with alanine residue were constructed by using the Quick-change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mutations were introduced using the following oligonucleotides: PM II (D158A, sense), 5′-CAACCATTTACATTTATTCTTGCTACAGGATCTGCTAATTTATGG-3′; PM II (D158A, antisense), 5′-CCATAAATTAGCAGATCCTGTAGCAAGAATAAATGTAAATGGTTG-3′; PM II (D338A, sense), 5′-GAAAAAGCAAACTGTATTGTAGCTAGTGGTACTAGTGCCATTACT-3′; PM II (D338A, antisense), 5′-AGTAATGGCACTAGTACCACTAGCTACAATACAGTTTGCTTTTTC-3′; PM IV (D155A, sense), 5′-CAACCATTTATGTTTATTTTCGCTACCGGTTCAGCTAATTTATGG-3′; PM IV (D155A, antisense), 5′-CCATAAATTAGCTGAACCGGTAGCGAAAATAAACATAAATGGTTG-3′; PM IV (D335A, sense), 5′-CAAAAAGCAAATGCTGTTGTTGCTAGTGGTACAAGTACTATAACA-3′; PM IV (D335A, antisense), 5′-TGTTATAGTACTTGTACCACTAGCAACAACAGCATTTGCTTTTTG-3′. The mutated sequences are underlined. The presence of the mutations was verified by sequencing, using an automated DNA sequencer (ABI prism).

Expression and Purification of Wild-Type and Mutant PM II and IV Proteins—Expression and purification of all recombinant PM II and IV proteins were conducted according to the method described by Hill *et al.* (*19*) with minor modifications. *E. coli* BL21 (DE3) transformed with wild-type and mutant forms of PM II and IV plasmids were induced for 5 h by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM. The cultured *E. coli* cells (0.5 liter of culture) were pelleted, resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.2), and lysed by sonication. Insoluble material containing the recombinant protein was pelleted and washed in 50 ml of buffer A (0.1 M Tric-HCl, pH 11.0, 50 mM β-mercaptoethanol). Insoluble material was re-pelleted, washed in 50 ml of buffer B (1 M urea, 0.1 M Tris-HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol), and washed again in 50 ml of buffer C (2 M urea, 0.1 M Tris-HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol). After centrifugation, the washed pellet was resuspended in 10 ml buffer D (6 M urea, 0.1 M Tris-HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol) and stirred overnight at 4°C to solubilize the recombinant protein. Residual insoluble material was removed by centrifugation, and the supernatant was diluted 1:10 in water and stirred for 5 h at room temperature to allow refolding of recombinant protein. The refolded solution was purified by HPLC on a Q Sepharose Fast Flow (Amersham Biosciences) equilibrated in 0.1 M Tris-HCl, pH 8.5. After extensive washing, the recombinant protein was eluted with a linear gradient of 0–0.9 M NaCl in the same buffer. The fraction containing the recombinant protein was concentrated and dialyzed in 10 mM Tris-HCl, pH 8.5, 5 mM NaCl, 20 mM β-mercaptoethanol. The purified protein was stored at −20°C before using it for any assay.

SDS-PAGE—Samples were subjected to 12% (for PM II and IV processing) and 15% (for hemoglobin degradation) SDS-PAGE according to Laemmli (*21*). Gels were stained with Coomassie Brilliant Blue R-250 (Sigma).

Processing Assays—Protease inhibitors were preincubated with wild type or mutant proteins of PM II and IV for 30 min at room temperature, and the mixture was acidified by the addition of one tenth volume of 1 M sodium acetate, pH 4.5, and incubated at 37°C. The resultant protein was resolved by 12% SDS-PAGE and then stained with Coomassie Blue.

PM Activity Assays—1. Hemoglobin degradation: Ten micrograms of human hemoglobin (Sigma) were incubated at 37°C for various times in the presence of recombinant enzymes had been pre-activated at 37°C for 30 min in 100 mM sodium acetate, pH 4.5. Digestion was terminated by the addition of SDS loading dye. The samples were boiled for 5 min prior to analysis by SDS-PAGE on a 15% polyacrylamide gel.

2. Fluorogenic substrate: The prodomains of PM II and IV (25 nM) were cleaved off by preincubation in an assay buffer (100 mM sodium acetate, pH 4.5, 10% glycerol and 0.01% Tween 20) at 37°C for 30 min. The reaction

was initiated by the addition of 3 μM FRET substrate (DABCYL-Glu-Arg-Nle-Phe-Leu-Ser-Phe-Pro-EDANS, AnaSpec Inc, San Jose, CA). After 30 min of incubation at room temperature, the fluorescence intensity (excitation 405 nm, emission 510 nm) was measured using a fluorescence microplate reader (Molecular Devices, USA). K_i values for recombinant PM II and IV were determined as described (*4*).

RESULTS

Processing of PM II and IV Is Inhibited by Pepstatin A—Recombinant PM II and IV of the four FV PMs expressed in *E. coli* are known to have comparable enzyme activities to native proteins (*4*, *19*). To investigate which protease inhibitors could prevent the processing of PM II and IV, they were expressed, refolded, and purified to homogeneity. Each recombinant proenzyme was incubated with various protease inhibitors for 30 min at room temperature and then activated by acidification to pH 4.5 for 1 h at 37°C. SDS-PAGE analysis revealed that the processing of PM II was inhibited by a common aspartic protease inhibitor pepstatin A (Fig. 1, upper panel), in agreement with the previous reports (*15*, *16*, *19*). Other inhibitors of serine (PMSF, leupeptin and DCI), cysteine (E-64, leupeptin and ALLN), or metalloproteases (EDTA, data not shown) had no effect on the processing of PM II. Likewise, the processing of PM IV was inhibited only by pepstatin A (Fig. 1, lower panel). Pepstatin A is known to inhibit aspartic protease activity by binding competitively in the active-site of the enzyme and then making hydrogen bonds with the two catalytic aspartic acids. Thus, our results suggest that, like the autoactivation of typical aspartic proteases, an intrinsic enzyme activity of PM II and IV is required for their autocatalytic processing.

Substrate Degradation by PM II and IV Is Inhibited by ALLN as Well as by Pepstatin A—Since hemoglobin degradation is an indicator of PM II and IV activities, we next examined the effects of various protease inhibitors on substrate (hemoglobin) degradation by PM II and IV. Each proenzyme acidified to pH 4.5 for 30 min at 37°C was preincubated with or without protease inhibitors for 30 min at room temperature, then incubated at 37°C in the presence of hemoglobin. Samples were removed after 1 h and analyzed by SDS-PAGE. As shown in Fig. 2A, hemoglobin degradation by PM II or IV was completely inhibited by 10 μM pepstatin A. Interestingly, PM II-mediated hemoglobin degradation was partially inhibited by 15 μM ALLN (40% inhibition), while the same concentration of ALLN completely blocked hemoglobin degradation by PM IV. Other protease inhibitors, including PMSF, had no inhibitory effects on hemoglobin degradation by PM II or IV.

To confirm the inhibitory effects of pepstatin A and ALLN on the activities of PM II and IV, each enzyme assay was performed with fluorogenic substrate. As shown in Fig. 2B, substrate degradation by PM II and IV was inhibited by pepstatin A in a dose-dependent manner. Complete inhibition of PM II and IV activities was shown

Fig. 1. **Effects of protease inhibitors on the processing of PM II and IV.** Each recombinant PM II and IV was incubated with or without protease inhibitors for 30 min at room temperature and then acidified in 100 mM sodium acetate, pH 4.5 for 1 h at 37°C. Protein samples were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. Lane 1 (without incubation), lane 2 (without inhibitor), lane 3 (pepstatin A, 10 μM), lane 4 (E-64, 10 μM), lane 5 (PMSF, 1 mM), lane 6 (leupeptin, 100 μM), lane 7 (DCI, 100 μM), lane 8 (ALLN, 15 μM), lane 9 (DMSO). P and M indicate the precursor and mature forms of PM, respectively.

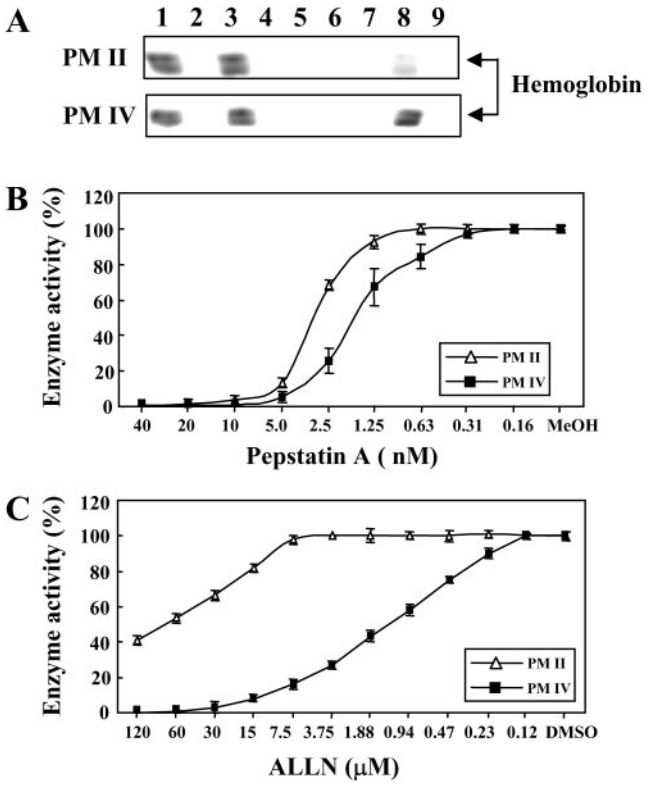

Fig. 2. **Effects of protease inhibitors on substrate degradation by PM II and IV.** (A) Effects of protease inhibitors on the degradation of hemoglobin by PM II and IV. Each recombinant PM II and IV (25 nM) was acidified in 100 mM sodium acetate, pH 4.5 for 30 min at 37°C, then preincubated with or without protease inhibitors for 30 min at room temperature, and finally incubated with hemoglobin (10 μg) for 1 h at 37°C. The degraded hemoglobin was analyzed by 15% SDS-PAGE and stained with Coomassie Blue. Lane 1 (without incubation), lane 2 (without inhibitor), lane 3 (pepstatin A, 10 μM), lane 4 (E-64, 10 μM), lane 5 (PMSF, 1 mM), lane 6 (leupeptin, 100 μM), lane 7 (DCI, 100 μM), lane 8 (ALLN, 15 μM), lane 9 (DMSO). (B and C) Effects of pepstatin A (B) and ALLN (C) on substrate degradation by PM II and IV. Each recombinant PM II and IV (25 nM) was acidified in an assay buffer (100 mM sodium acetate, pH 4.5, 10% glycerol and 0.01% Tween 20) and then incubated with the indicated concentrations of pepstatin A (B) or ALLN (C) for 30 min at room temperature. The enzyme-inhibitor complexes were incubated with 3 μM FRET substrate for 30 min at room temperature. Hydrolysis of the substrate was measured as the release of fluorescence in arbitrary fluorescence units (activity percentage of maximum). Error bars represent standard deviations of the results from three independent experiments.

at 20 and 10 nM of the inhibitor, respectively. However, there was a considerable difference in the inhibitory effects of ALLN on PM II and IV activities (Fig. 2C). Kinetic assessments revealed that ALLN is a noncompetitive inhibitor of PM II and IV with an IC_{50} of 60 and 1 μM, respectively. The inhibition rate constant K_i values for PM II and IV were 47.2 and 3.4 μM, respectively. These data, combined with the result that pepstatin A efficiently blocked the processing of PM II and IV (Fig. 1), suggest that pepstatin A inhibits both cis- and *trans*-cleavage activities of PM II and IV, whereas ALLN inhibits only their *trans*-cleavage activity of them. Moreover, sensitivities to ALLN are different.

Mutation of the Catalytic Asp Residue in Two Active-Site Motifs of PM II and IV Completely Inhibited Both Their Autocatalytic Processing and Proteolytic Activities—The inhibitory effect of pepstatin A on the processing of PM II and IV (Fig. 1) suggests that intrinsic enzyme activity is required for their autocatalytic processing. To confirm the importance of intrinsic enzyme activity for the processing of PM II and IV, mutants in two conserved active-site motifs of PM II (D158A and D338A) and IV (D155A and D335A) were constructed (Fig. 3A). Wild-type and mutant forms of recombinant PM II and IV proteins were expressed, refolded, and purified. SDS-PAGE analysis of these proteins revealed that all the mutant forms of PM II and IV were expressed at the same level as their wild-type proteins (Fig. 3B). As shown in Fig. 4, none of the mutant forms of PM II and IV were processed when the proteins were acidified to pH 4.5 and incubated for 1 h at 37°C, indicating the importance of intrinsic enzyme activity for the autocatalytic processing of PM II and IV.

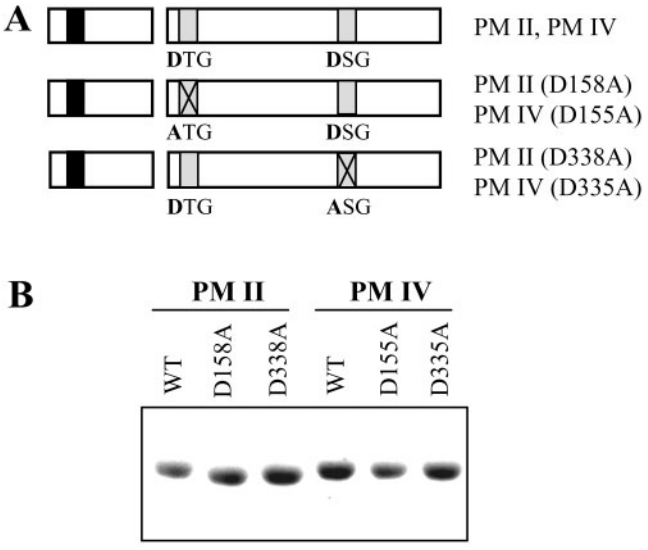

Fig. 3. **(A) Schematic diagrams of expression constructs of the active-site mutants of PM II and IV.** Each protein contains pro and mature regions. A transmembrane domain (black box) is present in the prodomain. The catalytic Asp (D) residues in two active-site motifs (shaded box) of the wild-type PM II and IV were replaced with an Ala (A) residue (crossed shaded box) as shown in schematically. (B) Expression and purification of the active-site mutants of PM II and IV. SDS-PAGE analysis revealed that all the mutant forms of recombinant PM II and IV proteins were expressed at the same level as their wild type proteins.

We next examined the proteolytic activity of mutant forms of PM II and IV towards hemoglobin. As expected, the active-site mutants of PM II and IV exhibited no enzyme activity against hemoglobin in 1 h of incubation, while wild-type PM II and IV efficiently degraded hemoglobin (Fig. 5, A and B). Also, when the proteolytic activity of wild type PM II and IV and their mutant forms was assessed with a fluorogenic substrate, the mutants showed no proteolytic activity (Fig. 5, C and D), further confirming the importance of active-site motifs of PM II and IV on their activity against substrate.

The processing of PM II and IV Occurs through an Intermolecular Autocatalytic as Well as a Transcatalytic Activity—Next, to investigate the detailed mechanism by which PM II and IV are processed, we used wild-type PM II and IV as enzymes and their mutant forms as substrates. When the active-site mutants of PM II, D158A and D338A, were incubated with wild-type PM II that had been activated by acidification, the mutant proteins were efficiently processed (Fig. 6A). Interestingly, wild-type PM IV processed the mutant forms of PM II as effectively as did wild-type PM II. Also, wild-type PM II showed similar *trans*-cleavage activity to wild-type PM IV in the processing of the mutant forms of PM IV (Fig. 6B). These results indicate that the processing of PM II or IV occurs by an intermolecular autocatalytic event as well as by a transcatalytic event between them.

To confirm whether both pepstatin A and ALLN could inhibit the *trans*-cleavage activity of wild type PM II and IV, we used the active-site mutants of PM II and IV as substrate. When activated wild-type PM II was incubated with the mutant form of PM II (D158A), the processing of mutant was completely inhibited by pepstatin A (Fig. 7, lane 3) but was partially blocked by ALLN (40% inhibition, lane 4), as in the case of substrate degradation (Fig. 2). Interestingly, both pepstatin A and ALLN completely inhibited the mutant processing by wild-type PM IV (Fig. 7, lane 7 and 8, respectively). In addition, the inhibitory effects of the two inhibitors on the processing of mutant PM IV (D155A) by wild-type PM II and IV were similar (data not shown). These results indicate that PM II is fully sensitive to pepstatin A but only partially so to ALLN, whereas PM IV is fully sensitive to both inhibitors.

DISCUSSION

Although the processing of PMs in the FV is critical for their proteolytic activity toward hemoglobin, which is

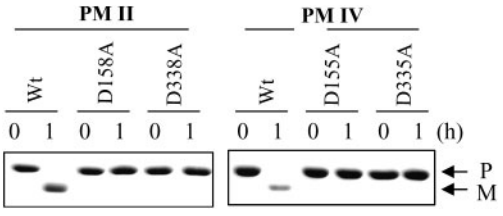

Fig. 4. **Processing of the wild-type and active-site mutants of PM II and IV.** Wild-type and mutant forms of recombinant PM II and IV proteins were acidified in 100 mM sodium acetate, pH 4.5 for 1 h at 37°C. Protein samples were analyzed by 12% SDS-PAGE and stained with Coomassie blue. P and M indicate the precursor and mature forms of PM, respectively.

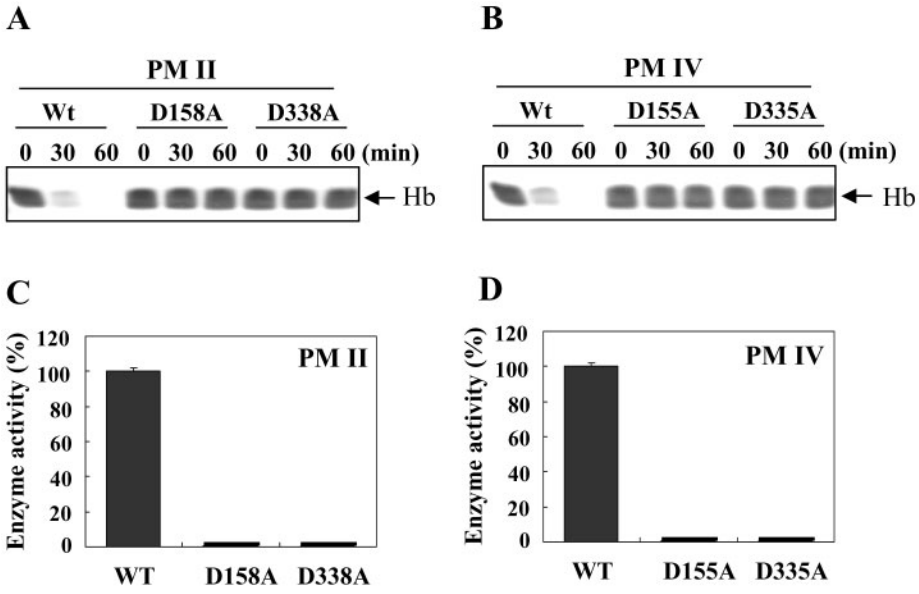

Fig. 5. **Proteolytic activity of wild-type and active-site mutants of PM II and IV.** (A and B) Hemoglobin degradation. Human hemoglobin (Hb, 10 μg) was incubated at 37°C for the indicated times with wild-type and mutant forms of recombinant PM II and IV proteins (25 nM) which had been acidified in 100 mM sodium acetate, pH 4.5 for 30 min at 37°C. The degraded hemoglobin was analyzed by 15% SDS-PAGE and stained with Coomassie Blue. (C and D) Fluorogenic substrate degradation. Wild-type and mutant forms of recombinant PM II and IV (25 nM) proteins which had been acidified in an assay buffer (100 mM sodium acetate, pH 4.5, 10% glycerol and 0.01% Tween 20) were incubated with 3 μM FRET substrate. Hydrolysis of the substrate was measured as the release of fluorescence in arbitrary fluorescence units (activity percentage of maximum). Error bars represent the standard deviations of the results from three independent experiments.

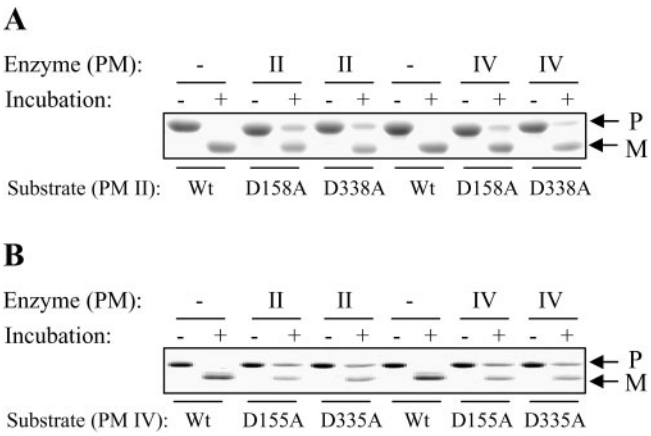

Fig. 6. **Conversion of the zymogen of active-site mutants of PM II or IV to the mature form by incubation with wild-type PM II or IV.** Recombinant active-site mutants of PM II (A, 4 μg) or IV (B, 4 μg) were incubated at 37°C in 100 mM sodium acetate, pH 4.5, for 30 min in the presence or absence of wild type PM II or IV (80 ng) at a protein ratio of 50:1. After 30 min of incubation, the samples were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. P and M indicate the precursor and mature forms of PM, respectively.

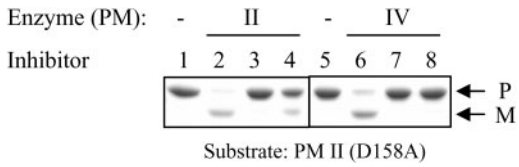

Fig. 7. **Effects of pepstatin A and ALLN on the conversion of the zymogen of active-site mutant of PM II to the mature form by incubation with wild-type PM II or IV.** Recombinant active-site mutant of PM II (D158A) was incubated at 37°C in 100 mM sodium acetate, pH 4.5, in the absence (lane 1 and 5, respectively) or presence of wild-type PM II and IV which had been acidified and preincubated without an inhibitor (lane 2 and 6) or with pepstatin A (10 μM, lane 3 and 7) or ALLN (15 μM, lane 4 and 8) for 30 min at room temperature. The ratio of wild-type PM II or IV to active-site mutant (D158A) was 1:50. After 30 min of incubation, the samples were analyzed by 12% SDS-PAGE and stained with Coomassie Blue. P and M indicate the precursor and mature forms of PM, respectively.

essential for the survival of the malarial parasite, the mechanism of their processing is not fully understood. In the present study, we have found that the prodomains of PM II and IV are cleaved not only by autocatalytic processing, which involves both intra- and inter-molecular steps, but also by transcatalytic processing between PM II and IV. In addition, we have shown that pepstatin A inhibits both *cis*- and *trans*-cleavage activities of PM II and IV, while ALLN inhibits only the just *trans*-cleavage activity.

PMs are synthesized as inactive precursors and converted into active enzymes in the FV by removal of the prodomains (*4*, *12*, *15*, *16*). Like typical aspartic proteases, recombinant PM II and IV were processed autocatalytically under acidic conditions in a pepstatin A–sensitive manner (Fig. 1), in agreement with the previous reports (*15*, *16*, *19*). However, based on the results that the processing of PMs *in vitro* or *in vivo* was blocked by several calpain inhibitors including ALLN but not by pepstatin A, the presence of a calpain-like protease for the processing of PMs has been suggested (*12*, *15*, *17*). We present here several lines of evidence that the processing of both PM II and IV occurred by autocatalytic and transcatalytic events between them, rather than by other proteases. First, pepstatin A blocked the processing of PM II and IV by inhibiting both their *cis*- and *trans*-cleavage activities (Figs 1 and 2), which indicates that the autocatalytic processing of PM II and IV is an intra- and inter-molecular process. Both PM II and IV have a conserved tripeptide D(T/S)G in two active sites found in other aspartic proteases such as cathepsin D and E (*4*, *22*, *23*). Mutations

in the two active-site aspartic residues of PM II and IV resulted in a complete loss of their processing (Fig. 4) and proteolytic activity (Fig. 5), as shown in cathepsin E (24). These data, combined with the effectiveness of pepstatin A (Fig. 1) support the notion that the processing of PM II and IV is autocatalytic. The involvement of an intramolecular process in the autocatalytic processing of both PM II and IV was supported by the result that ALLN did not block PM processing (Fig. 1), but efficiently blocked *trans*-cleavage activity of PM II and IV (Fig. 2). ALLN inhibits intermolecular processes but not intramolecular processes. The autocatalytic processing of PM II and IV *via* an intermolecular event was demonstrated by incubating the active-site mutants of PM II and IV as a substrate and their respective wild-type PMs as an enzyme, which resulted in a processing of both active-site mutants (Fig. 6). Second, we here provide direct evidence that the processing of both PM II and IV can be induced through *trans*-cleavage activity between PM II and IV. In the mixture of wild type PM IV and active-site mutant of PM II in a 1:50 molar ratio, or *vice versa*, mutant proteins were processed under acidic conditions (Fig. 6). Furthermore, there was no difference in *trans*-cleavage activities between wild-type II and IV on each other's mutant as substrate. These results apparently demonstrate that both PM II and IV serve as not only a substrate but also a protease (PM-convertase) for the processing of each other's proteins *in vitro*. Since the four FV PMs have a close similarity in structure and active-site (*4*), it is probable that PM I and HAP are also involved in PM processing with their *trans*-cleavage activity, as with PM II and IV. This possibility is now under investigation with recombinant PM I and HAP proteins.

Unlike other aspartic proteases, PM II and IV are sensitive to ALLN with different sensitivities: ALLN blocked the *trans*-cleavage activity of PM II and IV (Fig. 2) without inhibiting their *cis*-cleavage activity (Fig. 1). This atypical ALLN sensitivity of PM II and IV was further substantiated by incubating them as an enzyme with the active-site mutants of PM II as a substrate in the presence of ALLN, which resulted in a partial and complete inhibition of the processing of mutant protein by wild-type PM II and IV, respectively (Fig. 7). However, this unusual feature of both PM II and IV might explain in part why ALLN blocked the processing of PMs in a cell-free *in vitro* processing assay system (*12*). Considering both the partial inhibitory effect of ALLN on the *trans*-cleavage activity of PM II and its autocatalytic processing, the question remains of how PM II processing is sensitive to ALLN *in vitro* processing assay (*12*). One plausible explanation is that PM IV, which is highly sensitive to ALLN, may be mainly responsible for PM II processing rather than its autocatalytic processing. This possibility remains to be investigated. Furthermore, we cannot rule out the possibility that a calpain-like convertase which is sensitive to several calpain inhibitors, including ALLN (*12*), is responsible for the processing of PMs, until its putative single gene (*25*) is cloned and characterized.

Of four analogous PMs in the FV of *P. falciparum*, PM IV has been considered as the primary drug target because PM IV is the ortholog of the only food vacuole PM found in Plasmodium species that infect humans (*26*). However, two recent reports on a malarial parasite that lacks single or double food vacuole PM genes demonstrated their functional redundancy and the lack of need of a particular PM (*27*, *28*). Thus, a rational drug design focused on a single PM is unlikely to result in an effective antimalarial agent. A different approach to antimalarial drugs aimed at PMs can be to develop inhibitors of PM-convertase that processes PMs to their active form. However, our results strongly suggest that the processing of FV PMs is likely to be transcatalytic among PMs, which could rule out PM-convertase as a target for antimalarial drug design. Thus, it appears that good drug design strategies focused on PMs are to develop compounds capable of inhibiting all four of FV PMs, as reported in a recent work (*29*). It will be worthwhile to examine the processed level of each PM in the parasite which lacks a single, double or triple PMs to get more definitive information on their transcatalytic processing.

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